THE BRITISH JOURNAL OF
EXPERIMENTAL PATHOLOGY

VOL. XXV JUNE, 1944 NO. 3

EXPERIMENTAL STREPTOCOCCAL LESIONS OF THE RABBIT'S EYE AND THEIR TREATMENT.

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Received for publication April 27, 1944.

PREVIOUS work has shown that experimental infections of the cornea provide a suitable means of testing the effectiveness of various forms of therapy. Such a method has proved useful in investigating lesions produced by *Ps. pyocyanea*, *Staphylococcus aureus* and the pneumococcus (see Robson, 1944).

Haemolytic streptococci are comparatively uncommon in ocular infections in man, but are frequently found in infections of other parts of the body. Infections of the vitreous and aqueous with haemolytic streptococci have been produced (Rambo, 1938; Venco, 1939; Klein and Sorsby, 1943), but these lesions are not suitable for the accurate study of various forms of treatment. It seemed, therefore, desirable to produce corneal lesions similar in type to those which have been found suitable for the study of infections produced by other organisms.

METHODS.

Attempts were first made to infect the cornea, from which the epithelium had been removed over a small area, with a variety of strains of haemolytic streptococci, but these all proved unsuccessful. A number of these cultures were then injected into the cornea and two of them proved suitable for the production of experimental lesions, namely—

(1) A strain isolated from the throat of an early case of scarlet fever (Ic).

(2) A strain isolated from a septic wound (Scott).

The Ic strain usually produced more severe lesions than the Scott strain.

Certain unexpected results made it desirable to produce lesions with a strain known to be fully sensitive to sulphonamides, and the Richards strain was used after passage through mice to increase its virulence. The lesions produced by this strain were mild, and therefore not altogether satisfactory for the purpose of therapeutic investigations.

All organisms were grown in broth, and 18 to 24 hour cultures were used for injection into the cornea by the method previously described (Robson and Scott, 1943). Great care was taken to ensure that the blebs were of equal size in both eyes.

The following substances were used for treatment:

1. Penicillin solution. Two preparations were used, namely, (1) a solution of the

barium salt containing 65 Oxford units per c.c.; immediately before use this was converted into the sodium salt by the addition of the necessary amount of solid sodium sulphate, which precipitated the barium; and (2) a solution of the phosphated barium salt, containing 226 units per c.c., All solutions were stored in the refrigerator.

2. Penicillin ointment. This was made up according to a formula similar to that used by Clark, Colebrook, Gibson and Thomson (1943) in the treatment of burns. 10 g. of lanette wax was dissolved in 25 g. of castor oil and heated to 70° C.; 20 c.c. of water heated to 65° C. was added to the mixture, which was then allowed to cool somewhat; 50 c.c. of the solution of penicillin (containing 226 units per c.c.) was then thoroughly mixed in. The control ointment contained 10 g. lanette wax, 25 g. of castor oil and 70 c.c. of water.

3. Sodium sulphacetamide (albucid soluble). This was supplied to us as 30 per cent. solution, with a pH of 7·4.

4. Sulphanilamide, finely powdered and suspended in saline, in 10 per cent. and 30 per cent. suspension.

5. Microcrystalline sulphathiazole, supplied to us as a 15 per cent. suspension.

6. Sodium sulphadiazine. This powder was dissolved in saline to form a 30 per cent. solution with pH 9·6.

7. A solution containing 6·66 per cent. of diaminodiphenylsulphone; each molecule was in combination with two molecules of galactoside, and the solution also contained four molecules of free galactoside. This preparation is similar to Tibatin (described by Domagk, 1942).

8. Propamidine ointment 0·6 per cent. This had the following composition:

Propamidine	1 g.
Water	100 c.c.
Castor oil	36 g.
Lanette wax	20 g.

The lanette wax was dissolved in the castor oil and heated to 70° C.; the solution of propamidine in water was heated to 65° C. and added to the mixture.

9. Propamidine jelly, 0·1 per cent.

10. A detergent, Duponal M.E. dry, in concentrations of 0·1 per cent., was added to the sodium sulphacetamide, the sodium sulphadiazine solution, and the "Tibatin" solution in some experiments, in order to increase their penetration into the ocular tissues. Duponal M.E. (minimum of electrolyte) dry is derived from technical lauryl alcohol.

Treatment was started either immediately or at various intervals after inoculation. In the case of solutions and suspensions three drops were instilled at hourly intervals for 36 hours, and thereafter at hourly intervals from 9 a.m. to 5 p.m. for about a week. The control eyes were similarly treated with saline.

The ointments (penicillin and propamidine) were applied four times a day for about a week, and the control eye received a similar ointment without any active constituent.

The propamidine jelly was applied at the same intervals as the solutions. The control eyes in this experiment were untreated as no suitable control jelly was available.

The eyes were observed every day for the first few days, and subsequently at longer intervals. Photographs were taken usually at the third day, and also later in the experiment. The total period of observation was at least 7 days, and usually more than two weeks. A small percentage of the animals died during the first five days from septicaemia.

The severity of the lesions was evaluated according to an arbitrary scale. With

experience it has been possible to give numerical values to lesions (infiltrates, abscesses and ulcers) of various severities as follows :

1. *Slight.*—Small infiltrate, which does not form an abscess or ulcer, and leaves a small superficial scar.

2. *Moderate.*—Larger infiltrate which may form a small abscess and ulcer, and leaves a denser scar but not greater than 2·5 × 2·5 mm. in size.

3. *Severe.*—Infiltrates about 4 × 4 mm., going on to abscess and ulcer formation, with hypopyon. A large vascularized scar is left. This is described below as the typical severe lesion.

4. *Very severe.*—A lesion involving most of the cornea, but not going on to perforation. Very severe lesions were observed in only a few eyes.

All experiments were performed on groups of animals, and the severities of the lesions evaluated according to the above scale. As a rule one eye in each animal was treated and the other used as a control. The results for any particular set of eyes (treated or control) were averaged and the values so obtained are given in the tables. When the treatment was applied systemically, i.e. by injection, averages were taken of all the eyes in the group of animals.

RESULTS.

Course of control lesions.

The severity of the lesions varied with the different strains of streptococci, and also in different animals receiving the same strain, but was very similar in the two eyes of any one animal (in the absence of effective treatment applied to one eye).

The typical severe lesion produced by the Ic strain took from 5–7 days to reach its maximum severity. At 24 hours there was a slight to moderate infiltration of the cornea with a small degree of surrounding oedema. This was accompanied by moderately severe inflammation of the conjunctiva with chemosis and frequently with small haemorrhages, and fairly severe iritis.

During the next few days the infiltration spread, not infrequently giving off linear or branching processes, with increasing oedema which ultimately involved the whole cornea. Abscess formation was noted two or three days after inoculation. Loss of epithelium usually occurred over the whole area of infiltration, but ulceration was less extensive. The ulcers, sometimes multiple, appeared to be of a punched-out type though fairly shallow, and did not show the undermined edge typical of the pneumococcal ulcer. Hypopyon appeared about the third day, and vascularization of the cornea commenced two or three days later.

Healing started about the beginning of the second week and was slow ; ultimately a dense scar with a variable amount of vascularization was left. Occasionally a descemetocele was noted, but never perforation.

In milder lesions no ulceration, vascularization, or hypopyon occurred, and healing was much more rapid.

Effects of treatment.

(1) *Penicillin.*—These are summarized in Table I. When treatment was started at one hour the effect was very striking ; with one exception the resulting lesions were slight. Treatment with penicillin ointment, though beneficial, was less effective than with the solution. When treatment with the solution was delayed for 12 or 24 hours the effect appeared to be slight.

(2) *Sulphonamides.*—These are summarized in Table II. It will be seen that when the treatment is started one hour after inoculation, none of the sulphonamides

TABLE I.—*Treatment with Penicillin.*

Treatment.	Interval between inoculation and first treatment. (Hours.)	Number of animals.	Code numbers.	Average final corneal lesions.		Difference between treated and control eyes.	Strain of H.S.
				Treated eyes.	Control eyes.		
Penicillin solution :							
(*a*)	1	6	1033–1039	0·7	2·8	2·1	Ic
(*a*)	1	6	1082–1087	0·7	2·1	1·4	Scott.
(*b*)	1	3	1211–1213	0·5	2·1	1·6	Richard.
(*b*)	12	4	1107–1110	1·8	2·4	0·6	Ic.
(*b*)	24	5	1101–1105	1·5	2·0	0·5	Ic.
Penicillin ointment	1	3	1171–1173	1·5	2·8	1·3	Ic.

(*a*) : 65 units per c.c. (*b*) : 226 units per c.c.

TABLE II.—*Treatment with Sulphonamide.*

Treatment.	Interval between inoculation and first treatment. (Hours.)	Number of animals.	Code numbers.	Average final corneal lesion.		Difference between treated and control eyes.	Strain of H.S.
				Treated eyes.	Control eyes.		
30% Sulphanilamide	1	6	1055–1061	2·9	2·2	−0·7	Ic.
,,	1	6	1088–1093	2·7	1·5	−1·2	Scott.
,,	1	5	1205–1210	1·8	2·0	0·2	Richard.
30% sodium sulphacetamide	1	6	1040–1045	2·0	1·9	−0·1	Ic.
Ditto	1	4	1072–1075	2·9	3·4	0·5	Scott.
,,	1	4	1223–1226	1·8	1·6	−0·2	Richard.
15% microcrystalline sulphathiazole	1	5	1049–1054	2·8	2·3	−0·5	Ic.
Ditto	1	5	1200–1204	2·0	1·5	−0·5	Richard.
30% sodium sulphacetamide + 0·1% Duponal	1	6	1464–1469	1·8	3·3*	1·5	Ic.
30% sodium sulphadiazine + 0·1% Duponal	1	6	1470–1475	2·2	3·3*	1·1	Ic.
10–30% sulphanilamide	0	9	1233–1242 1256–1260	1·5	2·0	0·5	Ic.
30% sodium sulphacetamide	0	7	1234–1236 1262–1265	1·6	2·4	0·8	Ic.
Ditto	0	5	1384–1388	1·0	2·6	1·6	Ic†
,, + 0·1% Duponal	0	5	1384–1388	0·8	2·6	1·8	Ic†
Ditto	0	4	1434–1437	1·0	2·5	1·5	Ic‡
30% sodium sulphadiazine + 0·1% Duponal	0	4	1438–1441	1·5	2·6	1·1	Ic‡
"Tibatin" + 0·1% Duponal	0	5	1401–1405	2·1	2·6	0·5	Ic.

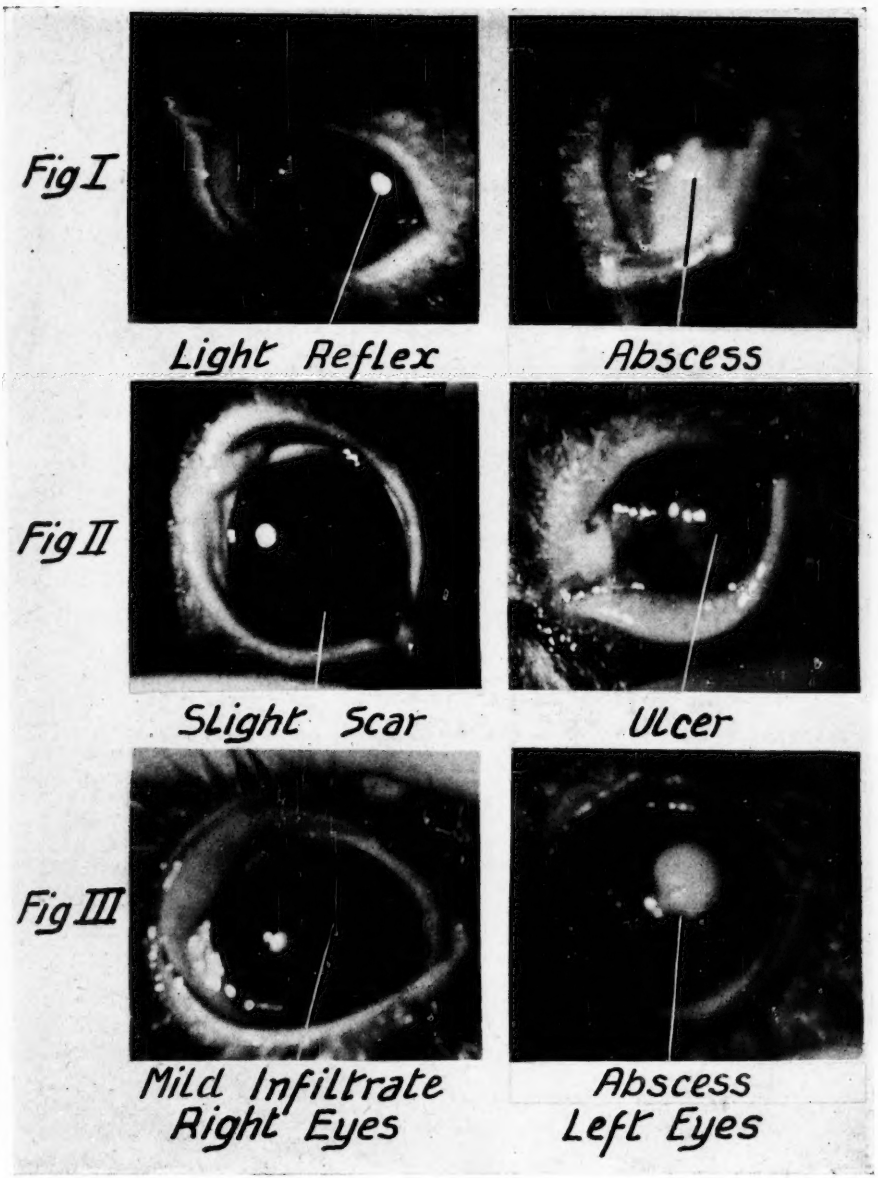
* Control eyes of animals 1464–1469. One set of eyes of 1470–1475 were treated with penicillin at 1 hour, and gave an average lesion of 1·0.

† See text.

‡ These two experiments were done at the same time.

FIGS. 1 and 2.—Illustrating the effect of treatment with penicillin applied to the right eye, and started 1 hour after inoculation. (Rabbit 1033 ; haemolytic streptococci, Ic.) Fig. 1 : Condition 24 hours after inoculation. Fig. 2 : Condition 20 days after inoculation.

FIG. 3.—Illustrating the effect of treatment, applied to right eye, with 30 per cent. sodium sulphacetamide + 0·1 per cent. Duponal M.E. dry, started 1 hour after inoculation. (Rabbit 1465; haemolytic streptococci Ic.) Condition 3 days after inoculation.



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used (sulphanilamide suspension, micro-crystalline sulphathiazole, sodium sulphacetamide) produced any beneficial effect.

When these results were first obtained, it appeared possible that the absence of activity was due to the strains used (Ic and Scott) being sulphonamide resistant. Colonel Colebrook kindly tested these two strains *in vitro* by the plate method, and found them to be sensitive. We also found them to be sensitive when tested by inoculation in mice. As a further precaution, we performed similar experiments on eyes with the Richards strain, known to be sulphonamide sensitive, and again obtained negative results when the treatment was delayed for one hour.

In all experiments in which sulphanilamide suspension was used there was evidence of a deleterious effect upon the ocular tissues. The area of loss of corneal epithelium in treated eyes was usually considerably greater than in the control eyes. In the latter, the staining with fluorescein usually covered an area little greater than that immediately involved by the focus of infection (infiltrate, abscess, or ulcer), whereas in the treated eyes large areas of the cornea remote from this focus were stained. In addition the conjunctivitis was frequently worse in the eyes treated with the sulphanilamide suspension. No such effects followed the repeated application of a 30 per cent. sodium sulphacetamide solution or a 15 per cent. microcrystalline sulphathiazole suspension.

When the treatment with sulphonamide was started immediately after inoculation some benefit was obtained. With 30 per cent. sodium sulphacetamide the results were definite, though not as good as with penicillin at one hour. In view of the marked irritant effects of a 30 per cent. sulphanilamide suspension as already noted, we used a 10 per cent. suspension in 5 of the animals in which treatment was started immediately, and 30 per cent. in the other 4. The irritant effect of the 10 per cent. suspension was much less. In both these groups of animals the beneficial effect was slight and approximately equal, and these 9 animals have therefore been considered together in Table II.

(3) *Detergent* (see Table II).—It has been shown that detergents increase the penetration of drugs, including the sulphonamides, into the ocular tissues. Experiments were therefore performed to determine whether the addition of a detergent to sulphonamides would increase their efficiency.

The addition of Duponal M.E. dry to the 30 per cent. solution of sodium sulphacetamide slightly increased its effectiveness when the treatment was started immediately. In this experiment the right and left eyes of 5 animals (1384–1388) were used for a direct comparison of the effect of Duponal, the left eyes of another 5 animals (1401–1405) being used as controls.

In each animal the eye in which Duponal was used was slightly better than the other eye; hence it would seem that the addition of the detergent to the sodium sulphacetamide increases its effect. Further evidence for this conclusion was obtained from the following experiment. In this, the treatment with 30 per cent. sodium sulphacetamide and Duponal was delayed for one hour, but nevertheless, produced a beneficial effect (see Table II).

In view of the high chemotherapeutic activity of sulphadiazine (Bell and Roblin, 1942) it seemed desirable to compare its effect with the other sulphonamides already used. Sodium sulphadiazine was combined with Duponal in order to obtain the maximum effectiveness. When the treatment with this was started immediately after inoculation, the results were not quite as good as those obtained by a combination of 30 per cent. sodium sulphacetamide and Duponal, though both treatments produced a beneficial effect (compare 1438–1441 with 1434–1437, which were all done at the same time). Similarly these two drugs, each in combination with Duponal, were

used in the treatment which started at one hour after inoculation. Here, too, the results with sodium sulphacetamide were, if anything, slightly better (compare 1464–1469 with 1470–1475, which were all done at the same time).

Domagk (1942) has recently reported that Tibatin (digalactoside of diaminodiphenyl sulphone) is highly effective in the treatment of experimental haemolytic streptococcal infections. We investigated a similar compound (see above) in combination with 0·1 per cent. Duponal. This treatment, started immediately, scarcely produced any beneficial effect (see Table II, animals 1401–1405).

(4) *Propamidine* (see Table III).—It has recently been suggested (Thrower and Valentine, 1943 ; McIndoe and Tilley, 1943 ; and others) that propamidine be used in the treatment of infected wounds. This substance, unlike the sulphonamides, is active in the presence of pus ; but, owing to its greater toxicity, comparatively low concentrations must be used. The results obtained with a 0·6 per cent. ointment, and a 0·1 per cent. jelly, both show that some beneficial effect can be produced, even when treatment is delayed for one hour (see Table III). But, in both experiments, the drug produced marked irritation, giving rise to the same ill-effects (i.e. conjunctivitis and loss of corneal epithelium) as those caused by the sulphanilamide suspension.

Relative effectiveness of local and general administration.

There has been some discussion as to whether sulphonamides should be used locally or generally in the treatment of the infections of the anterior parts of the eye and of the conjunctiva. A number of experiments were performed to investigate this question. The results are summarized in Table IV.

The dose of sulphonamide given by injection was in all cases 0·25 g. per kilo three times a day (i.e. 0·75 g. per kg. per day) for a week.

In the first experiment subcutaneous administration of sodium sulphapyridine was compared with local application of 30 per cent. sodium sulphacetamide solution. As can be seen from the Table, the local treatment was more effective.

It seemed possible that the comparative failure of the general treatment might be due to delay in absorption of the drug from the site of injection. In the second experiment, therefore, the first dose of sodium sulphapyridine was given intravenously, the following injections being subcutaneous. This treatment was, however, no more effective. Moreover, in this experiment, sodium sulphadiazine was administered to a group of animals by the same routes and in the same doses as the sodium sulphapyridine ; the results were exactly the same. In the animals treated locally the sodium sulphacetamide was combined with Duponal, while in another group of animals, sodium sulphadiazine with Duponal was applied locally. As can be seen in Table IV, the local treatment was consistently more effective than the systemic treatment.

In a third experiment the value of the general administration of another sulphonamide, sodium sulphathiazole, was tested. This also was given in doses of 0·25 g. per kg. three times a day, but the first treatment was delayed till one hour after inoculation. The treatment was entirely ineffective. As sulphathiazole does not penetrate well into the rabbit's eye (see Bellows, 1943), the experiments with this sulphonamide were not pursued.

As the above findings showed that the systemic administration of sulphonamides is less effective than their local application in haemolytic streptococcal infections, it seemed desirable to determine whether this would also hold for infections with other organisms. A similar investigation was therefore carried out on pneumococcal lesions (Type 19). In the fourth experiment sodium sulphapyridine was injected

TABLE III.—*Treatment with Propamidine.*

Treatment.	Interval between inoculation and first treatment. (Hours.)	Number of animals.	Code numbers.	Average final corneal lesions. Treated eyes.	Average final corneal lesions. Control eyes.	Difference between treated and control eyes.	Strain of H.S.
0·6% Propamidine ointment	1	6	1165–1170	1·6	2·2	0·6	Ic.
0·1% Propamidine jelly	1	4	1228–1231	1·1	1·7	0·6	Richard.

TABLE IV.—*Comparison of Local and Systemic Treatments.*

Number of experiment.	Treatment. Drug.	Treatment. Method of administration.	Interval between inoculation and first treatment. (Hours.)	Number of animals.	Code numbers.	Average final corneal lesion. Treated eyes.	Average final corneal lesion. Control eyes.	Difference between treated and control eyes.
1	Sodium sulphapyridine	Subcutaneous	0	5	1374–1383	2·0		0·6
	30% sodium sulphacetamide	Local	0	5	1384–1388	1·0	(a)	1·6
	Saline	„	0	5	1401–1405	(a)	2·6	..
2	Sodium sulphapyridine	Intrav. + subcu.	0	3	1426–1429	2·0		0·6
	Sodium sulphadiazine	Ditto	0	4	1430–1433	2·0		0·6
	30% sodium sulphacetamide + 0·1% Duponal	Local	0	4	1434–1437	1·0	2·5	1·5
	30% sodium sulphadiazine + 0·1% Duponal	„	0	4	1438–1441	1·5	2·6	1·1
3	Sodium sulphathiazole	Subcutaneous	1	5	1266–1270	2·4		−0·1
	Saline	Local	1	10	1256–1265	(b)	2·3	..
4	Sodium sulphapyridine	Intrav. + subcu.	1	8	1491–1498	2·4		1·4
	30% sodium sulphacetamide + 0·1% Duponal	Local	1	7	1499–1505	1·8	3·8	2·0

(a) See Table II for details of these eyes.
(b) Eyes treated with sulphanilamide and included in Table II.

In the first three experiments the infecting organism was the haemolytic streptococcus (Ic), and in the fourth experiment it was the pneumococcus (Type 19).

TABLE V.—*Effect of Duponal on the Local Effectiveness of 30 per cent. Sodium Sulphacetamide and 30 per cent. Sodium Sulphadiazine in Pneumococcal Lesions.*

Group.	Number of animals.	Code numbers.	Treatment.	Average final corneal lesions.	Organism.
1	6	1509–1514	R.E. 30% sodium sulphadiazine + 0·1% Duponal	2·1	Pneumococcus (Type 19).
			L.E. 30% sodium sulphadiazine	2·2	
2	5	1515–1520	R.E. 30% sodium sulphacetamide + 0·1% Duponal	2·3	„
			L.E. 30% sodium sulphacetamide	2·6	
3	3	1521–1526	R.E. 0·1% Duponal	3·9	„
			L.E. saline	4.1	

intravenously for the first dose and then subcutaneously in one group of rabbits, while 30 per cent. sodium sulphacetamide in combination with 0·1 per cent. Duponal was applied locally to the right eye of another group of rabbits, the left eyes being used as controls. As can be seen from Table IV, the local treatment was slightly more effective than the systemic.

Since it has previously been found that 10 per cent. and 30 per cent. solutions of sodium sulphacetamide are equally effective in the treatment of pneumococcal lesions, it seemed desirable to determine whether the detergent could be of any value in increasing the effectiveness of the 30 per cent. solution in pneumococcal lesions. The experiment shown in Table V was therefore performed. It is evident that the detergent had no appreciable effect; it did not produce any action when used by itself, nor did it increase the effectiveness of the two sulphonamides used.

DISCUSSION.

These experiments show that by the intracorneal injection of cultures of certain strains of haemolytic streptococci ocular lesions can consistently be produced which are suitable for the investigation of the action of various methods of therapy. In order to make certain that the developing lesions were actually due to this organism and not to secondary infection of the traumatized area by another organism, cultures were taken from developing infiltration sites just before the onset of ulceration and a pure growth of haemolytic streptococci was obtained.

The results clearly demonstrate the superiority of penicillin over the sulphonamides in the local treatment of such lesions. This is in agreement with previous findings on the relative value of penicillin and sulphonamides in experimental lesions due to the pneumococcus and *Staphylococcus aureus*.

As was expected, the results with sodium sulphacetamide are better than those with sulphanilamide. It has been demonstrated by Bell and Roblin (1942) and others that sulphanilamide has a lower chemotherapeutic activity than the other substances of this group commonly in use. The present experiments have, moreover, shown that suspensions of sulphanilamide can produce irritant effects, sometimes very appreciable, on the delicate tissues of the eye, and suggest that such suspensions or the powder itself are not suitable for repeated application in clinical practice.

Unexpected, however, is the finding that sodium sulphadiazine was not more effective than sodium sulphacetamide, since the former sulphonamide is, under certain circumstances, appreciably more active than sulphacetamide (see Bell and Roblin, 1942). Moreover, these two sulphonamides, applied locally, produced approximately equal effects not only in haemolytic streptococcal lesions, but also in pneumococcal infections of the cornea (Tables II and V). It is possible that these favourable results with sodium sulphacetamide are due to better penetration into the ocular tissues, a view supported by data discussed by Bellows (1943).

Bellows and Gutmann (1943) have recently discussed the use of wetting agents in increasing the penetration of sulphonamides into ocular tissues. The present experiments show that, under certain circumstances, a wetting agent can increase their therapeutic effect in ocular lesions. This is shown by the fact that a combination of sulphonamide with Duponal produces a beneficial effect on the streptococcal lesions when treatment is started one hour after inoculation, whereas none of the sulphonamides tried, including microcrystalline sulphathiazole, produced any such effect at that interval, when not combined with a wetting agent. Duponal did not increase the effectiveness of 30 per cent. sodium sulphacetamide or sulphadiazine in the pneumococcal infections, but previous experiments (Robson and Scott, 1943) have shown

that increasing the concentrations of sodium sulphacetamide from 10 per cent. to 30 per cent. does not appreciably increase the effectiveness of the treatment in such pneumococcal lesions; the failure of Duponal to increase the value of a 30 per cent. solution is thus not unexpected.

Propamidine was found to have appreciable chemotherapeutic actions, and in the present experiments compared favourably with the sulphonamides. But this drug also produced marked irritation, and on this account it seems doubtful if it is suitable for application to the sensitive ocular tissues.

In comparing the relative value of local and systemic treatment of experimental lesions with sulphonamides, sodium sulphapyridine was used for general administration in most experiments, since the concentrations of the drug in the ocular tissues are nearly as high as in the blood (see Bellows, 1943). In one experiment, however, sodium sulphadiazine was also used in a group of animals (Table IV, Experiment 2), since, though its concentration in the ocular tissues is appreciably lower than in the blood, its chemotherapeutic activity is greater than that of sulphapyridine. In all experiments the local application of the sulphonamide produced more favourable results than the general treatment. The difference was especially striking in the case of the lesions due to haemolytic streptococci. These results suggest that the local application of sulphonamides should play an important part in the prophylaxis and treatment of infections in the anterior part of the eye.

SUMMARY.

1. Methods are described for the production of experimental haemolytic streptococcus lesions of the cornea, suitable for investigating the action of chemotherapeutic substances.

2. The local application of penicillin produced a highly beneficial effect on the course of such lesions.

3. (*a*) The local application of sulphonamides produced no beneficial effect when an interval of one hour elapsed between the injection of the organisms and the first treatment. When the first treatment immediately followed inoculation, beneficial effects were produced.

(*b*) When the sulphonamide was combined with a detergent (0·1 per cent. Duponal M.E. dry) the beneficial effect was still obtained when treatment was delayed for one hour. Sodium sulphacetamide and sodium sulphadiazine produced approximately equal effects.

4. Propamidine produced a beneficial effect when treatment was delayed for one hour, but caused irritation.

5. The effectiveness of local and systemic treatment with sulphonamides was compared in lesions produced by haemolytic streptococcus and by pneumococcus (Type 19). The local treatment was appreciably more effective.

We are very grateful to Dr. Levinthal for his valuable suggestions and help in this work; to Colonel Colebrook for his advice and for testing the sulphonamide resistance of the strains. Dr. Maxted kindly supplied us with the Richard strain of H.S. We are also indebted to the W. H. Ross Foundation (Scotland) for the Prevention of Blindness, who have defrayed the expenses of the investigation; to Dr. Trevan, of the Wellcome Research Foundation, who kindly supplied us with penicillin made by Dr. Pope and Miss Stevens; to Dr. Hey, of British Schering Research Institute, for the "tibatin"; to Mr. Edwards, of British Schering Ltd., for sodium sulphacetamide (albucid soluble); to Messrs. Menley & James for microcrystalline sulphathiazole; and to Messrs. May & Baker for propamidine powder and jelly.

REFERENCES.

BELL, P. H., AND ROBLIN, R. O.—(1942) *J. Amer. chem. Soc.*, **64**, 2905.
BELLOWS, J. G.—(1943) *Arch. Ophthal.*, **29**, 888.
Idem AND GUTMANN, M.—(1943) *Ibid.*, **30**, 352.
CLARK, A. M. COLEBROOK, L., GIBSON, T., AND THOMSON, M. L.—(1943) *Lancet*, i, 605.
DOMAGK, G.—(1942) *Dtsch. Tierarz. Wschr.*, **50**, 301.
KLEIN, M., AND SORSBY, A.—(1943) *Brit. J. Ophthal.*, **27**, 241.
MCINDOE, A. H., AND TILLEY, A. R.—(1943) *Lancet*, i, 136.
RAMBO, V. C.—(1938) *Amer. J. Ophthal.*, **21**, 739.
ROBSON, J. M.—(1944) *Brit. J. Ophthal.*, **28**, 15.
Idem AND SCOTT, G. I.—(1943) *Brit. J. exp. Path.*, **24**, 50.—(1943) *Lancet*, i, 100.
THROWER, W. R., AND VALENTINE, F. C. O.—(1943) *Ibid.*, i, 133.
VE[illegible]CO.—(1939) *Ann. Ottal. clin. Ocul.*, **67**, 179.

TUMOURS OF THE THYROID PRODUCED BY 2-ACETYL-AMINO-FLUORENE AND ALLYL-THIOUREA.

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Received for publication May 5, 1944.

IN a previous communication (Bielschowsky, 1944) the localization and distribution of the tumours induced by 2-acetyl-amino-fluorene in the white rats of our colony has been described. The material at our disposal has increased considerably in the meantime, but so far not a single tumour of an endocrine gland has been found, nor is there any reference to a tumour originating from cells with internal secretion in the paper of Wilson, De Ed and Cox (1941), the discoverers of the carcinogenic action of this compound. One can therefore assume that these cells are far less susceptible to the carcinogenic action of the drug than the epithelial cells of many other organs, for instance of the liver or mammary gland. It seemed of interest to see if this natural resistance could be broken down, and to investigate the conditions under which tumours can be produced by 2-acetyl-amino-fluorene in endocrine glands. The thyroid was chosen for this experiment for the following reason: From human pathology it is known that cancer of the thyroid nearly always originates in a goitrous gland. Since goitre can be easily produced in rodents, the best experimental approach to the problem at hand seemed to investigate the action of the carcinogenic agent on a goitrous thyroid gland. The purpose of this paper is to describe the production of tumours of the thyroid by the combined action of allyl-thiourea and 2-acetyl-amino-fluorene.

When this investigation was started in 1942 only two papers dealing with the goitre-producing action of thioureas had been published (Kennedy, Richter and Clisby, 1942). During the previous year Kennedy, Purvis and Griesbach had described the effect of brassica seed diets on rats, and had shown that the pituitary plays a decisive role in the pathogenesis of this type of goitre. Kennedy (1942) advanced the hypothesis that a urea derivative might be the substance responsible for the brassica effect, and he found that allylthiourea produced goitre in rats. This substance seemed to be less toxic than phenyl-thiourea (Richter and Clisby, 1942), and was therefore chosen for

our investigation. After a few weeks of feeding this compound it became obvious that the doses employed by Kennedy were too high for long-term experiments, and it was found that not more than about 6 mg. daily of allylthiourea would be tolerated when the administration had to be continued for many months. This amount is of about the same order as the dose found effective by Astwood (1943). In a preliminary experiment it was also found that after feeding allyl-thiourea for 7–8 months the gland did not regain completely its normal structure, even when 100 days were allowed for the thyroid to recover.

METHODS.

On the basis of these experiences the following procedure was adopted: Acetyl-amino-fluorene was given, as in our previous experiments, at a daily dose of 4 mg. per rat for 30 weeks. The rats received daily 5 mg. of allyl-thiourea each for the first 4 weeks, and then 6 mg. daily until the animals were killed. Twenty rats (10 males, 10 females) were used. Ten received the standard diet (Bielschowsky, 1944), with the addition of acetyl-amino-fluorene and allyl-thiourea; 10 served as controls, and received the same diet with the addition of allyl-thiourea only. When an experimental animal had to be killed, generally because of the presence of a tumour, a control animal of the same sex was sacrified too. The animals consumed the diet completely.

RESULTS.

Table I summarizes the post-mortem findings of the ten experimental animals, Table II the histological findings of the thyroids.

TABLE I.

Duration of experiment.	Rat No.	Tumours in organs other than the thyroid.
197 days	3	Tumour of duct. acust. ext.
204 ,,	10	Adenocarcinoma of breast.
230 ,,	2	Malignant tumour of liver (mixed cholangioma-hepatoma); adenoma of lung; beginning carcinoma of the kidney.
237 ,,	7	Tumour of duct. acust. ext.
244 ,,	8	Nodular hypertrophy of liver; tumour of duct. acust. ext.; adenocarcinoma of breast.
259 ,,	9	Beginning malignant hepatoma; tumour of duct. acust. ext.
262 ,,	6	Nodular hypertrophy of liver; tumour of duct. acust. ext.
279 ,,	1	Malignant hepatoma; beginning tumour duct. acust. ext.
286 ,,	5	Malignant hepatoma.
286 ,,	4	Malignant tumour of the liver (mixed cholangioma-hepatoma).

Nos. 1–5 males, Nos. 6–10 females.

The thyroids of all animals were enlarged, and with the exception of two (Nos. 9 and 6), the glands of the control and the experimental animals appeared macroscopically very similar. The microscopic picture, however, was different in the two groups. Whereas the control animals showed only a uniform hypertrophy and hyperplasia with considerable loss of colloid, additional alterations were found in the thyroids

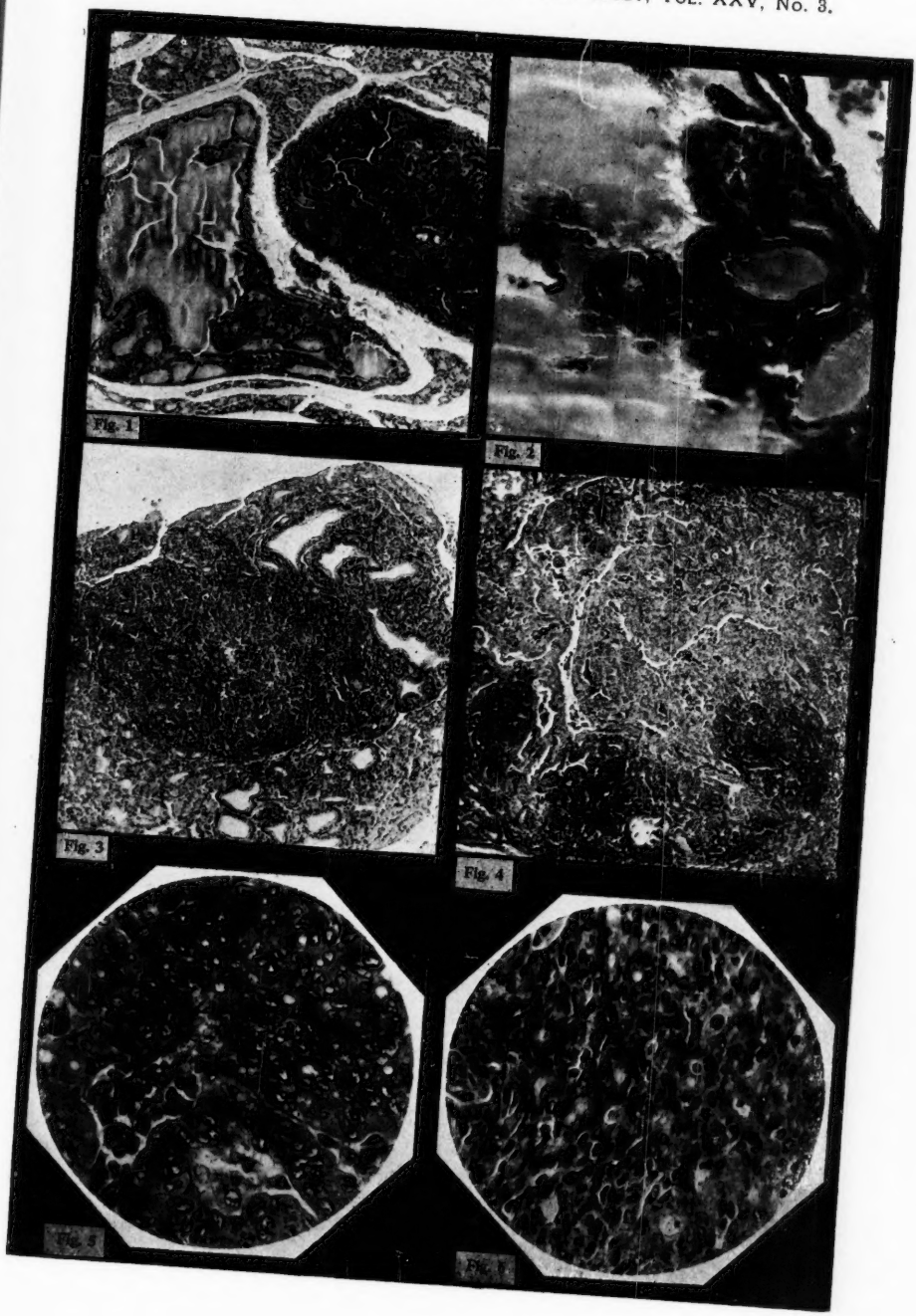
TABLE II.

Duration of experiment.	Rat No.	Thyroid gland.
197 days	3	Goitre ; hyperplasia with loss of colloid ; first signs of formation of adenomatous nodules.
204 ,,	10	Goitre ; hyperplasia with loss of colloid only.
230 ,,	2	Goitre ; hyperplasia ; several minute adenomata.
237 ,,	7	Goitre ; hyperplasia ; several minute admomata ; area of malignant growth.
244 ,,	8	Goitre ; hyperplasia ; multiple adenomas.
259 ,,	9	Goitre ; macroscopic nodule in left lobe (malignant adenoma) ; multiple adenomas.
262 ,,	6	Macroscopic tumour (adenocarcinoma).
279 ,,	1	Goitre ; hyperplasia ; multiple adenomas.
286 ,,	5	Goitre ; hyperplasia ; multiple adenomas.
286 ,,	4	Goitre ; hyperplasia ; multiple adenomas.

of nine out of the ten experimental rats. There are nodules which are easily distinguishable from the surrounding tissue, and which become visible to the naked eye in stained sections through their darker colour. In these nodules the epithelial cells are closer packed, they are of cuboid or cylindrical shape and their nuclei are compact and stained dark blue. Many of these nodules contain spaces which are filled with bright red stained colloid (Fig. 1). Into these cyst-like structures the epithelium proliferates in the form of papillomatous excrescences (Fig. 2). These nodules grow by expansion and compress the surrounding tissue when they reach a certain size. They are not encapsulated, nor does the stroma participate conspicuously in their formation. Their number and size varies considerably in our material ; in some animals only a few small nodules are found ; in others they are so numerous as to justify the term miliary. The larger ones measure 0·5 to 1·5 mm. diameter. The term adenoma must be applied to these newly-formed epithelial structures.

In three females further alterations of a different type were observed. In one animal (No. 7) a small area was found where the epithelium grew as a solid mass, the follicular glandular structure was completely lost and mitoses were frequent (Fig. 3). Serial sections revealed that this new growth, which infiltrated neighbouring tissue, originated in the periphery of an adenoma. In the two other cases macroscopically visible changes were present. The left lobe of the thyroid of rat No. 9 contained in its lower pole a small yellowish nodule of about 2 mm. diameter. A stained section revealed that it was not of uniform structure like the previously described adenomata (Fig. 4). In some parts the alveolar arrangement was more or less preserved, but the cells contained large clear nuclei of irregular size and shape (Fig. 5). In other parts the epithelium was arranged in tubules formed by a kind of syncytium of large cells with nuclei of varying size, while at the periphery the epi-

FIG. 1.—× 85. Typical adenoma from preliminary experiment.
FIG. 2.—× 400. Proliferation of epithelium into colloid cyst. Rat No. 8.
FIG. 3.—× 85. Area of malignant growth. Rat No. 7.
FIG. 4.—× 85. Malignant adenoma. Rat No. 9.
FIG. 5.—× 400. Central area from malignant adenoma. Rat No. 7.
FIG. 6.—× 400. Area from periphery of malignant adenoma. Rat No. 7.
FIG. 7.—× 5. Adenocarcinoma of Rat No. 6.
FIG. 8.—× 85. Penetration of the capsule. Rat No. 6.
FIG. 9.—× 400. Area of increased stroma. Adenocarcinoma. Rat No. 6.
FIG. 10.—× 400. Central area of adenocarcinoma. Rat No. 6.
FIG. 11.—× 85. Thyroid of control rat after 244 days of administration of allyl-thiourea.



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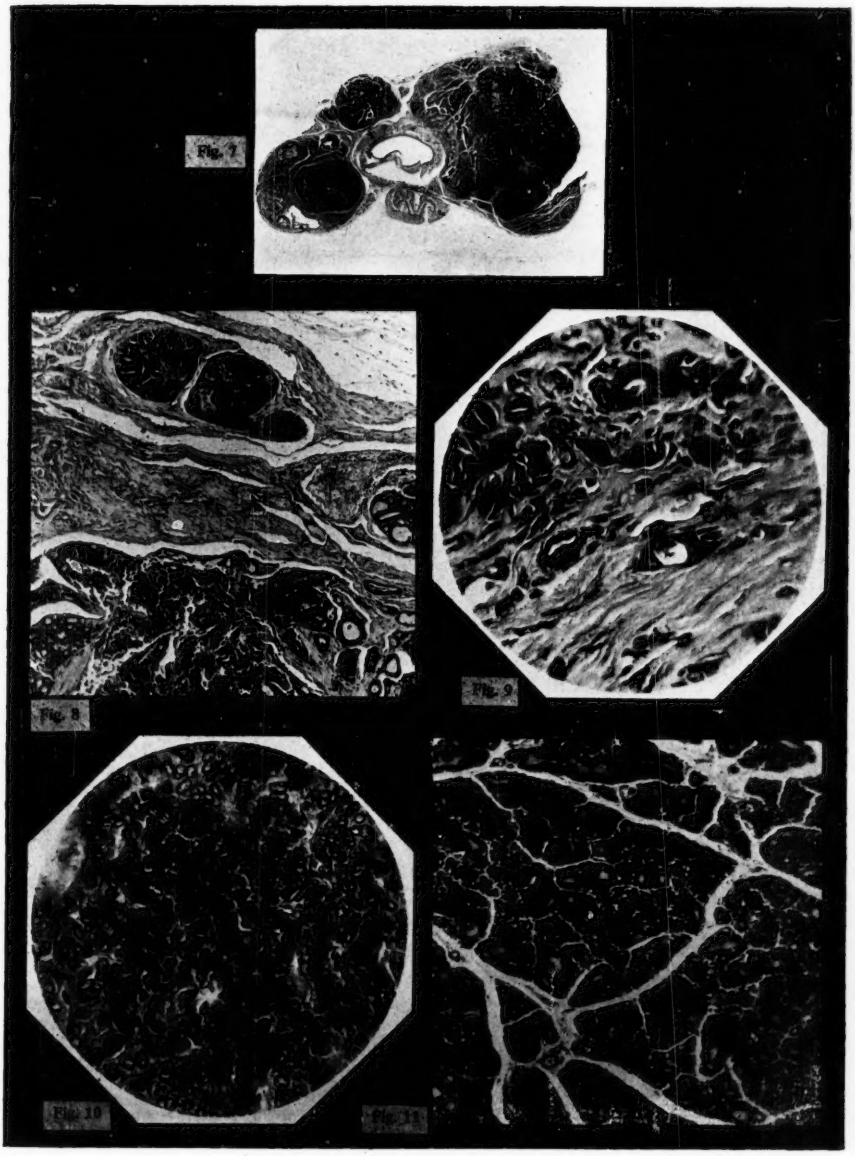

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thelium grew in a completely disorganized fashion (Fig. 6). In this last region the epithelial cells became more basophilic and mitoses were frequent. In Rat 6 the thyroid gland was considerably enlarged and had lost its normal shape ; it was transformed into an irregular mass (10 by 8 by 5 mm.) ; the right lobe was specially prominent, and the tissue firm and indurated (Fig. 7). The histological study revealed an adenocarcinoma which had penetrated the capsule (Fig. 8) at many points. Fundamentally the same changes were present as in No. 9, only in a more advanced stage. Where the alveolar arrangement was still present and colloid formed it stained blue in haematoxylin-eosin sections. There were large areas where the glandular structure was completely lost and the stroma increased, with signs of beginning hyaline degeneration present (Fig. 9).

DISCUSSION.

The results described above show that benign and malignant tumours of the thyroid have been produced by the combined action of allyl-thiourea and 2-acetyl-amino-fluorene. Neither drug when given alone induces neoplastic growth in the thyroid gland of the white rat. Prolonged administration of allyl-thiourea produces only a parenchymatous solid goitre ; 2-acetyl-amino-fluorene does not affect normal thyroid tissue at all. The stimulated hyperplastic thyroid is, however, susceptible to the carcinogenic action of acetyl-amino-fluorene, and tumours appear in an organ in which malignant growth has—as far as we know—only once been produced by chemical means (Esmarch, 1942). There is a striking difference between the great number of publications on experimental goitre of rodents and the paucity of papers on induced tumours of the thyroid. Hartwell's survey of compounds which have been tested for carcinogenic activity does not contain a single quotation of a chemically induced malignant tumour of the thyroid gland. He refers, however, to Yoshida's (1932) observation of metaplasia in the thyroid of guinea-pigs which received injections of ortho-amino-azo-toluene as " very similar to epithelioma."

Wegelin described in 1927 the histological changes present in the thyroids of a colony of white rats in which goitre was endemic. The animals were fed on cooked oats and corn with the addition of some bread, a diet which produced rickets and hypertrophy of the parathyroid glands in some animals. Small doses of iodine prevented the formation of the diffuse parenchymatous goitre, but did not restore completely the normal histological structure of the gland. Wegelin reported that in contrast to his previous experiences more than 50 per cent. of the goitrous glands contained adenomata. His description of these nodules and the illustrating photomicrographs make it clear that his histological findings are very similar to the changes obtained in our experiment. He observed also one malignant struma (sarcoma) in an old rat. Hellwig (1935), who kept his rats on a very similar iodine-deficient diet to which calcium chloride was added, observed enlargement of the thyroid, and two of these hyperplastic glands contained single large adenomata ; no malignant growth was discovered. Esmarch (1942) obtained in 6 out of 10 rats malignant tumours of the thyroid by direct application of methylcholanthrene (3 sarcomas and 3 squamous carcinomas). Spontaneous tumours of the thyroid are so extremely rare in rats that experimental results are safe from this possible source of error.

There exists general agreement between statisticians, pathologists and surgeons that adenomatous goitre is a main factor in the origin of human cancer of the thyroid. Recently Goetsch (1943) showed that " apparently primary carcinoma in exophthalmic goitre is in fact secondary to malignant degeneration of an intermediate pre-existing minute adenoma," i.e. this rare thyroid tumour has the same starting-point. It is therefore clear that the appearance of an adenoma in a goitrous gland represents the

fundamental event in the pathogenesis of cancer of the thyroid. From the material submitted in this paper one must assume that the same process is responsible for the development of the malignant changes in the thyroids of our rats. But the crucial question remains, to which mechanism is the formation of the adenomata due ? The control rats show diffuse hyperplasia, which tends to become less pronounced the longer the experiment lasts. In animals which were kept alive for 40–41 weeks the follicles contained, especially at the periphery, more colloid than was found in rats sacrificed earlier. It seems that the effect of the drug tends to fall off. This may be due to an exhaustion of the continuously stimulated cells of the thyroid, to an acquired specific resistance of these cells, to an exhaustion of the cells of the pituitary which produce the thyrotropic hormone or to the formation of an antihormone. It appears that the cells of the adenomata are no longer under the control of the thyrotropic hormone, since abundant colloid is present in these nodules. The preliminary experiment already mentioned provided material which proves that these adenomata do not regress when the stimulation ceases. In one female rat which was killed 100 days after the withdrawal of both compounds a large nodule of typically adenomatous structure was found (Fig. 1).

No explanation can be given yet as to why the stimulated goitrous thyroid is susceptible to the tumour-producing action of 2-acetyl-amino-fluorene. Morphologically the stimulated epithelial cells are larger than normal thyroid epithelium; functionally they have lost the ability to synthesize thyroxine (Astwood *et al.*, 1943). Increased cell size is a factor which favours mitosis, and is sometimes one of the first signs of a precancerous condition—for instance in adenomatous nodules of the liver. It is possible that the carcinogenic agent only hastens a process which may come about in a hyperplastic gland "spontaneously." The time factor is very evident in Wegelin's observations; first endemic goitre appeared in his rat colony, and later his older animals developed adenomata of the thyroid. From here to the formation of malignant tumours is only one step, which, as human pathology has shown, Nature makes only too frequently. On the other hand, there can be no doubt that hormonal stimulation is a powerful factor in the pathogenesis of certain types of cancer. The development of cancer of the breast in mice and rats is dependent to a very large extent on the action of oestrogens. It must be left to further experiments to provide additional facts, and so help eventually to a better appreciation of the importance of the different factors involved in the pathogenesis of the tumours of the thyroid.

SUMMARY.

The production of benign and malignant tumours of the thyroid by the combined feeding of allyl-thiourea and 2-acetyl-amino-fluorene is described.

It is a pleasure to express my gratitude to Prof. H. N. Green and Dr. H. E. Harding for their interest and encouragement. My thanks are also due to Mr. O. Thornburn for valuable technical help, and to Mr. A. W. Collins for the care with which he prepared the photomicrographs.

This investigation was carried out under the aegis of the Yorkshire Branch of the British Empire Cancer Campaign.

REFERENCES.

ASTWOOD, E. B.—(1943) *J. Pharmacol.*, **78**, 79.
Idem, SULLIVAN, J., BISSELL, A., AND TYSLOWITZ, R.—(1943) *Endocrinology*, **32**, 210.
BIELSCHOWSKY, F.—(1944) *Brit. J. exp. Path.*, **25**, 1.
ESMARCH, O.—(1942) *Acta path. microbiol. scand.*, **19**, 79.

GOETSCH, E.—(1943) *Ann. Surg.*, **118**, 843.
GRIESBACH, W. E.—(1941) *Brit. J. exp. Path.*, **22**, 245.
Idem, KENNEDY, T. H., AND PURVES, H. D.—(1941) *Ibid.*, **22**, 249.
HARTWELL, J. L.—(1941) Federal Security Agency, U.S. Pub. Health Service.
HELLWIG, C. A.—(1935) *Amer. J. Cancer*, **23**, 550.
KENNEDY, T. H., AND PURVES, H. D.—(1941) *Brit. J. exp. Path.*, **22**, 241.
KENNEDY, T. H.—(1942) *Nature*, **150**, 233.
RICHTER, C. P., AND CLISBY, K. H.—(1942) *Arch. Path.*, **33**, 46.
WEGELIN, C.—(1927) *Schw. med. Wschr.*, **8**, 848.
WILSON, R. H., DE ED, F., AND COX, A. J.—(1941) *Cancer Res.*, **1**, 595.
YOSHIDA, T.—(1932) *Arch. path. Anat.*, **283**, 29.

THE DIFFERENCE IN CARCINOGENICITY BETWEEN SHALE OIL AND SHALE.

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Received for publication May 22, 1944.

A PREVIOUS investigation on the carcinogenic constituents of shale oil (Berenblum and Schoental, 1943) gave the following results: (1) The detection of 3 : 4-benzpyrene in blue shale oil; (2) the induction of tumours of the skin in mice, with a fraction of the oil that was entirely free from benzpyrene; and (3) the failure to detect benzpyrene in extracts of the original shale. These results suggested that the carcinogenic constituents of shale oil probably belonged to the same class of compounds as those in coal-tar (though obviously in very different relative amounts), and that, as in the case of coal-tar, their presence was due to the effects of destructive distillation (pyrolysis).

It is well known that coal (the natural material from which coal-tar is derived by destructive distillation) is devoid of carcinogenic action. By analogy, and in the light of the above results, it was thought likely that shale (the natural material from which shale oil is derived) would also be non-carcinogenic. The present investigation was undertaken to determine whether this was the case.

EXPERIMENTAL.

A sample of solid shale was finely ground, and continuously extracted with boiling chloroform for about a week. In order to avoid excessive heating, the extracted material was removed at frequent intervals, and fresh chloroform introduced. When freed from the chloroform, the extracted material was found to be a dark brown product of the consistency of soft wax, representing about 1 per cent. of the powdered shale. This product was diluted with benzene to a semi-liquid (tarry) consistency, and applied in this form to the skin of 20 white mice, in the inter-scapular region, at half-weekly intervals. The mice used were from a similar stock to those used for the carcinogenicity tests for shale oil and its fractions.

Eight of the 20 mice died from intercurrent infection between the 4th and 5th week of the experiment, but the remaining 12 mice bore the painting very well; all 12 were

still alive after 20 weeks of painting ; 10 after 30 weeks ; and 7 after 40 weeks. Painting is being continued on these 7 survivors (see Appendix).

The painted skin showed some slight thickening and loss of hair, both being less marked, however, than those following painting with shale oil. None of the animals, after 40 weeks of painting with *extract of shale* have developed tumours (benign or malignant) at the site of application. In contrast to these negative results, when *shale oil* was painted at half-weekly intervals for only 18 weeks, 6 out of 10 mice developed tumours at the site of application, the first tumours (papillomas) appearing in 2 mice at the 7th week of painting. All these tumours grew progressively in size, and at least one of them became malignant by the 18th week of painting.

CONCLUSIONS AND SUMMARY.

A sample of blue shale oil was found to be strongly carcinogenic to the mouse's skin, whereas a concentrated extract of shale (the natural product from which shale oil is obtained by retorting) failed to produce any tumours after 40 weeks of painting.

These results confirm the view, previously expressed by the authors (1943), on the evidence of fluorescence analysis of chromatographic fractions of shale oil and extract of shale, that the carcinogenic constituents of shale oil do not exist in the original shale, but owe their presence to pyrolytic effects during the retorting.

Appendix.—Final result of application of extract of shale: 5 mice alive after 45 weeks, 3 after 50 weeks, all dead after 51½ weeks; no tumours at site of application.

We are indebted to Scottish Oils Ltd. for generous supplies of blue shale oil and shale. We wish to thank Mr. H. W. Wheal for valuable technical assistance.

REFERENCE.

BERENBLUM, I., AND SCHOENTAL, R.—(1943) *Brit. J. exp. Path.*, **24**, 232.

THE PRODUCTION OF PENICILLINASE BY ORGANISMS OF THE *SUBTILIS* GROUP.

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Received for publication May 22, 1944.

PENICILLINASE, an enzyme destroying penicillin, was first described in certain bacteria by Abraham and Chain (1940). The enzyme, which was present in crushed bacterial suspensions, was of low activity. Harper (1943) described a method of preparing the enzyme from a paracolon bacillus. The active enzyme was present in acetone dried suspensions of the bacteria and was moderately active, 1·0 mg. destroying 200 units of penicillin in 12 hours. Harper has included this enzyme preparation in culture media, but it suffers from the disadvantage of not being dissociable from the bacterial bodies, so that sterilization by filtration is impossible. Lawrence (1943) described the destruction of penicillin by certain commercial enzyme preparations

of fungoidal origin, taka diastase and clarase. Later (1944) he stated that the activity was due to a soluble enzyme produced by contaminating organisms of the *subtilis* group. Because of the need for a soluble, easily sterilizable preparation for routine sterility tests on penicillin batches, and for the examination of bacteriological specimens containing penicillin-sensitive organisms and penicillin, a further study was undertaken.

METHODS AND RESULTS.

(a) *Organisms used.*—The highest yield of extracellular penicillinase was obtained with organisms of the *subtilis* group. Two of these, N.C.T.C. 6276 and 6346, were obtained from the National Collection of Type Cultures, and were the only strains of six *subtilis* strains received which showed marked penicillinase activity. Other strains used were a *subtilis*-like organism (S.T.A.) obtained from the air, and a class demonstration strain of *B. subtilis*.

(b) *Culture vessels and media.*—The organisms were seeded in 0·5 c.c. amounts from fully-grown broth cultures into 4-oz. medical flats containing 20 c.c. of Lemco broth, and were cultured with the bottles horizontal on their broad sides. If the bottles were shaken they were plugged with rubber bungs, through which passed short pieces of glass tubing lightly packed with wool, and allowing free communication between the air inside and outside the bottles. If cultured at rest ordinary wool plugs were sufficient. At the end of incubation the broth was neutralized, shaken to reduce its viscosity, centrifuged, and passed through a Seitz filter.

(c) *Estimation of penicillinase content.*—Estimations of penicillinase were made by comparison with an arbitrarily selected penicillinase solution, using a variation of the ring technique for penicillin estimation described by Heatley (1944). The procedure was as follows: Falling dilutions of the solution to be tested and of the standard enzyme solution at 1/200 and 1/300 were each added to equal volumes of penicillin (strength 25 Oxford units per c.c.) in M/15 phosphate buffer pH 7·0. The mixtures were incubated for 30 minutes at 37° C. and samples were pipetted into porcelain rings on staphylococcal-seeded agar plates, samples of the standard enzyme and penicillin mixture being included in each plate. The plates were left for 2 hours at 4° C., followed by 15 hours at 37° C. Zones of inhibition were matched against those of the standard enzyme solution, which usually gave rings of 17 to 20 c.mm. at the 1/200 dilution. Enzyme activity fell off gradually with dilution, typical zone inhibition values for the standard being as follows:

1/100 = 0 mm.; 1/150 = 12 mm.; 1/200 = 18 mm.; 1/300 = 23 mm.; 1/400 = 25 mm.; 1/500 = 26·5 mm., the last denoting little or no activity.

Dilution values given represent, not final dilutions, but those added to an equal volume of penicillin, 25 units per c.c. The method suffers from the disadvantage that the enzyme acts continuously on the substrate during and after its diffusion into the agar, so that all time values must be adhered to rigorously. Measurements made on the same day using the same batch of inoculated agar plates gave very close agreement between zones given by similar enzyme substrate mixtures on different plates, and on ten successive days the standard enzyme solution 1/200 and penicillin, 25 units per c.c., gave the following average values: 18, 12, 17, 12, 18, 19, 20, 18, 20, 19 mm. By repeating the test in doubtful cases no difficulty was experienced. A maximal yield of penicillinase, some 50 per cent. stronger than that present in the culture arbitrarily selected as a standard, was obtained under a variety of cultural conditions given in Table I. Minor variations in the various culture conditions listed have usually decreased penicillinase production by the organisms.

Table I.

Batch No.	*Subtilis* strain.	Penicillin addition. Mode of addition.	Time between seeding and first addition.	Total added.	Temperature.	Shaken.	Culture time.
(1) III 5	S.T.A.	500 u. 12-hourly	4 hours	2000 u.	37°C.	+	3 days
(2) III 7	„	125 u. 12-hourly	4 „	500 u.	„	+	3 „
(3) VIII 8	„	500 u. followed by 1000 u. 12-hourly	None	3500 u.	„	+	3 „
(4) IX 5	N.C.T.C. 6346	Ditto	„	4500 u.	„	+	3 „
(5) IX 6	N.C.T.C. 6276	„	„	„	„	+	3 „
(6) IX 10	Class	„	„	„	„	+	3 „
(7) IX 11	„	„	„	„	„	—	3 „
(8) IX 12	„	250 u. followed by 500 u. 12-hourly	„	2250 u.	„	+	3 „
(9) IX 5	N.C.T.C. 6346	1000 u. followed by 500 u. 24-hourly	7 hours	2000 u.	26°C.	+	2 „
(10) X 7	Class	Ditto	7 „	„	„	+	2 „
(11) X 9	„	2 of 1,000 u. 24-hourly	7 „	„	„	—	3 „
(12) XV 7	N.C.T.C. 6346	2 of 1,000 u. 48-hourly	4 days	„	„	—	9 „

20 c.c. Lemco broth seeded with 0·5 c.c. amounts of fully grown *subtilis* cultures.

Under the above experimental conditions the maximum penicillinase content was reached in every case. In column 7, + indicates that the culture was shaken continuously during incubation; — indicates that it remained at rest; u. = Oxford units of penicillin.

Enzyme activity in terms of penicillin destroyed was estimated as follows: 0·4 c.c. amounts of Lemco broth, seeded with staphylococci and containing 25 units of penicillin per c.c., were mixed with falling dilutions of standard pencillinase solution. After 12 hours growth had occurred in the tube containing a final dilution of 1/640 of enzyme, denoting a destruction of 16,000 units in 12 hours by 1 c.c. of the standard enzyme. By the agar plate method used for enzyme estimation the standard destroys only 400 × 11·5 units of pencillin in 15 hours, i.e. 4600 units per c.c., since the penicillin diffuses out more rapidly than does the enzyme.

(d) *Temperature of incubation.*—As can be seen in Table I, equally good enzyme yields have been obtained at 26° C. and 37° C. One exception was the *subtilis* organism No. 6276 obtained from the National Collection of Type Cultures, which gave a maximum at 37° C. only.

(e) *Effect of gas mixtures.*—No favourable effect was observed using mixtures of oxygen and air and of oxygen and carbon dioxide over the culture media.

(f) *Influence of continuous agitation.*—In general the only effect of continuous shaking using a method similar to that employed by Casman (1940) has been to give quicker growth with earlier maximal titres when penicillin was added during the growth phase. In Experiments 9–11, Table I, the optimum was reached in 3 days without shaking and in 2 days with shaking. A 10-fold increase was experienced in the last 24 hours of the unshaken culture. Evidently a stable enzyme, such as penicillinase, does not deteriorate in culture, and rapid growth fails to increase the final enzyme concentration as in the case of more labile enzymes.

(g) *Effect of penicillin additions.*—Penicillin added to the cultures increased the yield about 20- to 30-fold, as in the case of other adaptive enzymes (Dubos, 1940). An unusual feature of the adaptation was that the substrate might be added several days after the beginning of culture, when growth had presumably ceased in cultures

grown at rest at 26° (Table I, Experiment 12). Since penicillin added during the growth phase may inhibit the organisms partially or completely, and may fail to exert a maximum stimulatory effect as in many experiments not recorded, the following procedure used in Experiment 12 was finally adopted as a routine. 20 c.c. of Lemco broth in a 4-oz. medical flat plugged with wool, seeded with 0·4 c.c. of a fully-grown culture of No. 6346 *B. subtilis* from the National Collection of Type Cultures, is grown at 26° C., the bottle on its flat side. Penicillin, 1000 Oxford units in 0·4 c.c., is added on the 4th and 6th days of incubation, and the cultures shaken gently for a few minutes by hand. On the 9th day the medium is made neutral with acid, and, after centrifuging, filtered through a 3·6 cm. Seitz filter. 1/600 of this culture in seeded staphylococcal broth containing 25 units penicillin per c.c. should permit growth to occur in 12 hours at 37° C.

(h) *Stability of penicillinase.*—A 1/100 solution of the standard non-dialyzed penicillinase preparation was made in 0·14 M. NaCl, and the pH adjusted to various values between pH 4·0 and pH 9·0 by the addition of small amounts of buffer. Heated for 4 hours at 60°, there was no loss in activity between pH 5·0 and pH 7·0, a 50 per cent. loss at pH 8·0, and a 75 per cent. loss at pH 4·0 and pH 9·0. Boiling for 20 minutes at neutral pH caused no alteration in activity.

(i) *Partial purification of penicillinase.*—Some degree of purification was obtained by dialysis against 0·07 M. NaCl or distilled water, following concentration by pressure dialysis using a cellophane membrane. Details are given in a previous paper (1939). No loss was observed following dialysis against the salt solution, but against distilled water there was a 60 per cent. loss in 48 hours. Filtration of these dialysed solutions in 20 c.c. amounts through a 3·6 cm. Seitz filter pad showed a 60 per cent. loss by absorption, whereas undialyzed solutions showed no change. A concentrated dialysed penicillinase preparation having an activity exactly ten times the standard preparation, contained 2 mg. non-dialyzable material per c.c., corresponding to an enzyme activity of 80,000 Oxford units destroyed per mg. in 12 hours.

Use of Penicillinase in Routine Culture Media.

As described in the previous section, a penicillinase preparation of maximum titre or at least equivalent to the standard permits staphylococci to grow in 12 hours when added in a final dilution of 1/640 to seeded broth containing 25 units of penicillin per c.c. Under the same conditions pneumococci showed good growth in serum broth in 12 hours at a 1/80 dilution of penicillinase, and moderate growth at 1/160. At 24 hours the staphylococci had grown at a penicillinase dilution of 1/1280 and the pneumococci at 1/640. Assuming 20 units per c.c. as the maximum amount of penicillin likely to be present in a bacteriological specimen, and that 0·05 c.c. (10 large loopfuls) may be added to 10 c.c. of broth, 1/20,000 of a good penicillinase culture filtrate should be adequate and 1/4000 should give a wide margin of safety in ordinary liquid media. This is also adequate for blood cultures containing 1·0 Oxford units of penicillin per c.c. of blood added to 10 c.c. medium, at least 5 times the maximum blood level found in practice.

To determine the amount required for solid media, the standard penicillinase in falling dilutions was added to 2·5 per cent. horse blood agar at 56° C., plates being poured after 5 minutes. Large loopfuls (5 c.mm. of broth cultures of *Staphylococcus aureus*, *S. haemolyticus* and pneumococcus, all of average sensitivity) were added to 0·4 c.c. amounts of penicillin (25 units per c.c.) and after five minutes plated in 5 c.mm. amounts on the penicillinase plates. Table II shows that while staphylococci grew in 24 hours at 1/20,000 penicillinase in the agar, 1/2000 was needed for all three

organisms. It would therefore be best to employ a 1/500 concentration of the standard preparation in solid media.

TABLE II.

Final concentration of standard penicillinase in medium.	*Staph. aureus.*	*Str. haemolyticus.*	*Str. pneumoniae.*
1/2,000	Growth	Growth	Growth
1/4,000	,,	,,	*Nil*
1/8,000	,,	*Nil*	,,
1/20,000	Weak growth	,,	,,

Effect of varying dilutions of standard penicillinase in blood agar, on growth of organisms from liquid containing 25 units of penicillin per c.c. Volume plated = 5 c.mm. Period of growth 24 hours.

SUMMARY.

1. A stable extracellular penicillinase is produced in good quantity by organisms of the *subtilis* group in liquid media, if penicillin be added during or at the end of the growth phase.

2. Methods of determining enzyme activity and of the amounts required for routine culture media are described.

The work has been carried out under a grant made by the Nuffield Provincial Hospitals' Trust. I am very grateful to Professors H. W. Florey and A. D. Gardner for laboratory facilities, and to Pte. P. Bowdery, A.T.S., for valuable technical assistance.

REFERENCES.

ABRAHAM, E. P., AND CHAIN, E.—(1940) *Nature*, **146**, 837.
CASMAN, E. P.—(1940) *J. Bact.*, **40**, 601.
DUBOS, R. J.—(1940) *Bact. Rev.*, **4**, 1.
DUTHIE, E. S., AND CHAIN, E.—(1939) *Brit. J. exp. Path.*, **20**, 417.
HARPER, G. J.—(1943) *Lancet*, ii, 569.
HEATLEY, N. G.—(1944) *Biochem. J.*, **38**, 61.
LAWRENCE, C. A.—(1943) *Science*, **98**, 413.—(1944) *Ibid.*, **99**, 14.

THE NATURE AND MODE OF ACTION OF STAPHYLOCOCCUS COAGULASE.

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Received for publication May 30, 1944.

THE phenomenon of staphylococcus coagulase activity was first reported by Loeb in 1903 and, although its significance and practical importance were not appreciated for several years thereafter, the coagulase test is now generally accepted as affording the best single criterion of potential pathogenicity. The association of pathogenicity and power of coagulase production may, of course, be merely fortuitous, but a causal relationship is at least possible, so that the elucidation of the mode of action of coagulase in the clotting of plasma is desirable. None of the hypotheses suggested hitherto provides a satisfactory explanation of the species specificity of coagulase activity, the failure of occasional samples of human plasma to clot, and the non-coagulability of human fibrinogen which has been purified by repeated precipitation. The demonstration that staphylococcus coagulase requires activation by a secondary factor which is present in some plasmas, but absent from others, rendered these anomalies intelligible and forms the basis of the present report.

PRELIMINARY OBSERVATIONS.

Filterability of coagulase.

An analysis of the factors concerned in the reaction demanded the employment of coagulase free from living organisms. The literature contains many conflicting reports of filterability, doubtless because of the widely different adsorptive capacities of the filters used (Gross, 1931; Gengou, 1933; Vanbreuseghem, 1934; Walston, 1935). With Gradocol membranes of known porosity (Elford, 1931) we obtained regular and consistent results.

A typical experiment is illustrated in Table I. A strain of *Staph. pyogenes* was

TABLE I.—*The Filterability of Coagulase through Gradocol Membranes.*

	Final dilutions of filtrates tested with 1 in 10 dil. human citrated plasma.						
	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.
0·76μ filtrate .	+	+	+	+	+	+	—
0·50μ ,, .	+	+	+	+	+	+	—
0·31μ ,, .	+	+	+	+	+	—	—
0·11μ ,, .	—	—	—	—	—	—	—

+ = clot. — = no clot formation in 24 hours at 37° C.

grown in flasks of digest broth at 37° C. for 4 days. After centrifugation the supernatant fluid was clarified by filtration through paper pulp and then filtered through a 0·76μ membrane. Portions of this bacteriologically sterile 0·76μ filtrate were passed through other membranes of various pore sizes. All the filtrates were tested, in serial twofold dilutions, against a 1 in 10 dilution of citrated human plasma. Mixtures were kept in a water-bath at 37° C. and final readings were taken at 24 hours.

The results show that highly active sterile filtrates can be obtained with membranes of suitable porosity. The reduced potency of the 0·31μ filtrate and the total retention of active material by 0·11μ membranes indicate that coagulase is either itself a particulate substance, or associated with particulate material of fairly uniform size.

Thermostability of coagulase.

The results of thermostability tests are given in Table II. Phials containing active filtrate were totally submerged in water or oil baths for the required lengths of time and then rapidly cooled. The findings of Gross (1933) and Walston (1935) that filtrates retain considerable activity after heating for ½ hour at 100° C. are confirmed; in addition it is evident that heating at 80° C. causes some loss, that complete inactivation is rapid at 120° C., and that at 100° C. there is a steady progressive inactivation.

TABLE II.—*Thermostability of Coagulase.*

Heat treatment of filtrate.	Final dilutions of filtrates tested with 1 in 10 dil. human citrated plasma.							
	1/3.	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.
56° C. 30 min.	+	+	+	+	+	+	+	—
80° C. 30 ,,	+	+	+	+	+	+	—	—
100° C. 3 ,,	+	+	+	+	+	+	—	—
100° C. 30 ,,	+	+	+	+	+	—	—	—
100° C. 60 ,,	+	+	+	—	—	—	—	—
110° C. 10 ,,	+							
120° C. 10 ,,	—							
Unheated control	+	+	+	+	+	+	+	—

+ = clot. — = no clot in 24 hours at 37° C.

This relative thermostability allows the concentration of filtrates by distillation at 60 to 70° C. under reduced pressure. By this method we have obtained a ten-fold concentration together with some degree of purification.

Concentration and partial purification can also be obtained by precipitation with alcohol and by dialysis. Isolation of the active principle has not yet been attempted.

THE ROLE OF A SECONDARY FACTOR IN COAGULASE ACTIVITY.

The effect of secondary factor on the "non-coagulable" plasmas of certain species.

Although it is advisable to use human plasma in routine coagulase tests, rabbit or horse plasma are employed in many laboratories with apparently satisfactory results. Guinea-pig plasma however cannot be used, and several other species also have non-coagulable plasmas. A concentrated coagulase which clotted both human and rabbit citrated plasmas at a dilution of 1 in 800 completely failed to clot guinea-pig, mouse and fowl plasmas within 24 hours at 37° C., even at a dilution of 1 in 10. Vanbreuseghem (1934), however, found that guinea-pig plasma will coagulate if the test is carried

out at 20° C. instead of 37° C. We confirmed this, although the coagulation was slow and large doses of coagulase were required; on the other hand, mouse and fowl plasmas failed to clot at any temperature.

The obvious suggestion that susceptibility or non-susceptibility to coagulase action must be due to chemical or structural differences between the fibrinogens of different species led us to investigate the interchangeability of all the factors concerned in the normal coagulation of blood. The mechanism of blood coagulation was thoroughly elucidated by Mellanby (1908, 1909, 1930, 1933), who devised methods for the preparation of all the reagents concerned: fibrinogen, prothrombin, thrombin kinase and calcium chloride. Although serological tests show that chemical differences must exist, we found that, amongst the species mentioned above, these factors were freely interchangeable for the conversion of any of the fibrinogens to fibrin clot. The use of testis extracts as a source of kinase, however, provided the key to the problem of coagulase action. The experiments summarized in Table III show that the addition of either human or rabbit testis extract to guinea-pig, mouse or fowl plasmas renders them just as fully susceptible to coagulase as are human and rabbit plasmas.

The testis extracts were made by grinding the tissue with distilled water to make a 10 per cent. suspension, centrifuging out the coarser particles and clarifying the supernatant fluid through paper pulp. A guinea-pig testis extract was also found to contain activator substance but only in relatively small quantity for, in its presence, coagulase took several hours longer to clot guinea-pig plasma than when human or rabbit testis was employed. The fact that the guinea-pig extract proved to be just as rich in thrombokinase for the activation of normal prothrombin in the presence of calcium provided the first indication that coagulase activator is a substance distinct from thrombokinase; this was more fully established in later experiments. Human and rabbit sera may also be used for the supply of activator substance.

TABLE III.—*The Effect of Secondary Factor on the Behaviour of Different Plasmas.*

	Dilutions of coagulase.						
	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	Saline control.
Human plasma . . .	+	+	+	+	+	—	—
,, . ,, + testis extr.	+	+	+	+	+	—	—
Guinea-pig plasma . .	—	—	—	—	—	—	—
,, ,, + testis extr. .	+	+	+	+	+	—	—
Mouse plasma . . .	—	—	—	—	—	—	—
,, ,, + testis extr.	+	+	+	+	+	—	—
Fowl plasma . . .	—	—	—	—	—	—	—
,, ,, + testis extr. .	+	+	+	+	+	—	—

The effect of secondary factor on "non-coagulable" human plasma.

Several workers have reported that occasional samples of human plasma fail to clot with staphylococcus strains known to be coagulase-positive. The natural tendency has been to attribute such behaviour to the presence of anti-coagulase immune bodies, but all attempts to produce the antibodies experimentally have been unsuccessful (Gross, 1931; Walston, 1935), and Cruickshank (1937) found that the plasmas of persons who had recently recovered from severe staphylococcal infections were normally coagulable. Our own attempts to immunize rabbits and guinea-pigs against coagulase

have likewise been abortive. A deficiency of activator substance, however, in the non-coagulable plasmas would account for their abnormal behaviour, and experimental verification of this hypothesis was readily obtained.

Routine tests on 40 samples of human plasma revealed one only which failed to clot. This was then tested against seven different strains of coagulase positive staphylococci, and proved to be non-coagulable with all of them even after standing overnight at room temperature subsequent to the routine incubation for 3 hours at 37° C. There is thus no doubt that it would have been accepted by previous workers as a non-coagulable plasma. Nevertheless its non-coagulability was found to be relative rather than absolute. In Table IV it will be seen that a coagulase filtrate, effective against normal human plasma up to a dilution of 1 in 640, clotted the atypical plasma at a dilution of 1 in 40, but failed to do so at 1 in 80 even in 48 hours. Moreover, within the range of coagulase concentrations effective for both plasmas, a very marked lengthening of clotting time over the normal is evident in the case of the atypical plasma. These results indicate a deficiency, but not a complete absence, of activator substance in such abnormal human plasmas. It is interesting that a sufficiently large dose of coagulase (1 in 10 dilution of the preparation used in this particular experiment) compensates for the deficiency of activator, and so eliminates the differences between clotting times given by smaller doses. The addition of testis extract, by providing sufficient activator, causes the atypical plasma to behave like the normal in every respect. It also reduces the coagulation times of a normal plasma with smaller doses of coagulase, indicating that even normal plasmas do not contain activator substance in optimal amount for all doses of coagulase.

TABLE IV.—*Deficiency of Activator as the Cause of the Abnormal Behaviour of Atypical Human Plasma.*

(A) *Titration of Coagulase Filtrate on Typical and Atypical Plasmas.*

	Dilutions of coagulase.						
	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	1/1280.
Typical plasma . .	+	+	+	+	+	+	—
Atypical plasma . .	+	+	—	—	—	—	—

(B) *Effect of Addition of Testis Extract.*

	Typical plasma. Dilutions of coagulase.			Atypical plasma. Dilutions of coagulase.		
	1/10.	1/50.	1/250.	1/10.	1/50.	1/250.
Plasma + saline . . .	5	25	70	5	90	—
,, + testis extr. 1/10 dil.	5	20	45	5	23	—
,, + ,, ,, 1/5 ,,	5	15	30	5	15	90
,, + ,, ,, 1/2·5 dil.	5	10	20	5	15	35

+ = Clot formation. — = No clot in 48 hours' incubation at 37° C. Figures show clotting time in minutes.

Reduction of clotting time of diluted human plasma by addition of activator.

Other factors being constant, the greater the dilution of human plasma employed the longer is the coagulation time. The experiments summarized in Table IV suggested that the reason for this must be reduction of activator substance by dilution. The hypothesis was tested by the experiment shown in Table V. The dose of coagulase

used clotted a 1 in 50 dilution of human plasma, but only after overnight incubation, whereas the same dose together with a small quantity of testis extract clotted the 1 in 50 dilution actually in less time than coagulase alone took to clot the standard 1 in 10 dilution. The addition of testis extract was not without some effect even on the more concentrated plasma.

TABLE V.—*Reduction of Clotting Time of Dilute Plasma by Addition of Activator.*

	Dilutions of citrated human plasma.	
	1/10.	1/50.
0·2 c.c. coagulase	20 min.	Overnight
0·2 c.c. coagulase + 0·05 c.c. testis extr.	9 ,,	14 min.

The coagulation of purified fibrinogen-prothrombin complex.

Mellanby has shown that a fibrinogen-prothrombin complex can be extracted from plasma by dilution in distilled water and acidification with dilute acetic acid (Mellanby, 1908). It is apparently impossible by this method to obtain the fibrinogen free from prothrombin, but the removal of practically all kinase is demonstrated by the failure of the complex to clot on the addition of calcium chloride, and its rapid coagulation on the further addition of a little kinase in the form of tissue extract or fresh serum. Coagulase alone is entirely without effect on such purified fibrinogen-prothrombin because of the removal of all activator substance. Fibrinogen prepared by other methods such as precipitation with ammonium sulphate retains associated activator substance and also kinase unless several reprecipitations are carried out. This has led some workers to suppose that the progressive diminution of coagulase activity on fibrinogen, as the latter is progressively purified, is due to denaturation of the protein by the successive salt precipitations. Table VI illustrates a typical experiment showing the ready coagulability of purified fibrinogen by either the normal blood-clotting mechanism or the action of coagulase, provided that the essential constituents are supplied in each case. The testis extract serves as a source of either kinase or activator substance, but we have good evidence that the two are not identical. The clotting of purified fibrinogen by activated coagulase is more rapid than the clotting of plasma; even so the action of kinase in the presence of calcium chloride is still more rapid. Possible reasons for this are discussed below.

TABLE VI.—*Clotting of Fibrinogen by Activated Coagulase.*

Mixtures.	Coagulation time (minutes).	Reason for observed result.
0·2 c.c. FP + 0·2 c.c. $CaCl_2$ + 0·8 c.c. saline	—	Absence of kinase.
0·2 c.c. FP + 0·8 c.c. rabbit testis extract + 0·2 c.c. saline	—	Absence of $CaCl_2$.
0·2 c.c. FP + 0·2 c.c. coagulase + 0·8 c.c. saline	—	Absence of coagulase activator.
0·2 c.c. FP + 0·2 c.c. coagulase + 0·8 c.c. testis extract	4	Clotting by activated coagulase.
0·2 c.c. FP + 0·8 c.c. testis extract + 0·2 c.c. $CaCl_2$	2	Clotting by normal thrombin.

$CaCl_2$ = 0·6 per cent. solution. FP = fibrinogen-prothrombin complex. — = complete absence of clot.

The time factor in coagulase activation.

The experiments so far described support the view that coagulase is the precursor of a thrombin-like substance; it is comparable with prothrombin in that it requires activation by a substance analogous to kinase, but with the important difference that calcium ions are not essential for the activation. The time lag which occurs before clotting takes place, even with large doses of coagulase, suggests that the conversion of the coagulase to activated coagulase is a slow process compared with the activation of prothrombin by kinase and calcium chloride. The progressive formation of thrombin-like product can be readily demonstrated by incubating a mixture of coagulase and activator, and testing samples withdrawn at intervals upon citrated guinea-pig plasma. Guinea-pig plasma is particularly suitable for such tests because its content of activator substance is negligible. The results of such an experiment are shown in Table VII. Neglecting losses due to the thermolability of activator and thrombin-like product, each sample withdrawn must contain exactly the same quantities of coagulase and activator, either separate or combined into thrombin-like product, but the proportions of free and combined reagents vary and depend upon the length of incubation prior to sampling. The clotting times are a valid index of the amounts of thrombin-like product at the times of sampling because, subsequent to the addition of a sample to plasma, further combination of coagulase and activator is relatively extremely slow. Thus, the immediate sample which may be assumed to consist entirely of uncombined reagents took 6 hours to produce sufficient combined product for clotting, whereas a sample withdrawn only two minutes later caused clotting in 4½ hours because of its content of preformed thrombin-like substance. The reasons for slow combination in the final plasma mixtures are the decrease of concentration of reagents by the increase of volume and the effect of a colloidal menstruum. Retardation of plasma coagulation by each of these factors separately has been demonstrated experimentally.

TABLE VII.—*Progressive Formation of Thrombin-like Substance in a Mixture of Coagulase and Activator.*

Solution A = 2 c.c. coagulase + 0·3 c.c. human testis extract. Mixture incubated at 37° C.

0·2 c.c. samples of A withdrawn at intervals and tested on citrated guinea-pig plasma.

Sampling time.	Clotting time.
Immediate	6 hours.
2 minutes	4½ ,,
5 ,,	4 ,,
10 ,,	3½ ,,
35 ,,	2 ,,

The thermolability of activator substance and of thrombin-like product.

Unlike coagulase, both the activator substance and the thrombin-like product formed by their interaction are thermolabile. Two tubes, each containing the same mixture of coagulase and rabbit testis extract, were left for an hour at room temperature to allow formation of thrombin-like product. One tube was then heated for ½ hour at 56° C. Tests with 0·2 c.c. samples on diluted guinea-pig plasma gave the following results:

Unheated mixture at end of 1 hour. Clotted plasma in 25 minutes.
,, ,, ,, 1½ hours ,, ,, 23 ,,
Heated mixture (1 hr. at R.T. + ½ hr. at 56° C.) failed to clot plasma in 10 hours.

A similar experiment, but with a potent extract of human testis and heat treatment at 45° C., gave clotting times of 11 minutes and 10 minutes for the unheated mixture and 24 minutes for the heated mixture, indicating that some destruction of thrombin-like product occurs even at this lower temperature. This thermolability is probably due to destruction of the activator constituent of the product because, when coagulase and testis extract were heated separately at 56° C. for ½ hour and then mixed and left at room temperature for an hour, a sample failed to clot plasma in 10 hours.

The correlation of time, temperature and concentration factors.

The peculiar behaviour of guinea-pig plasma in failing to clot in routine coagulase tests at 37° C. whilst clotting in tests carried out at about 20° C. has been productive of much speculation and experimentation along lines which we believe to be fallacious. The low content of guinea-pig plasma in activator substance, coupled with the thermolability of both activator and thrombin-like product, provides a satisfactory explanation supported by every experimental test so far applied. The factors of concentration of reagents, time and temperature are, of course, mutually interdependent, and the exact share taken by each of them in the final result is difficult of analysis because of the lack of pure, standardizable reagents. An attempt at such an analysis has been made in many experiments similar to the one illustrated in Fig. 1. Identical mixtures of coagulase and human testis extract were made in two tubes, one of which was held at 37° C. and the other at room temperature. From each tube two small

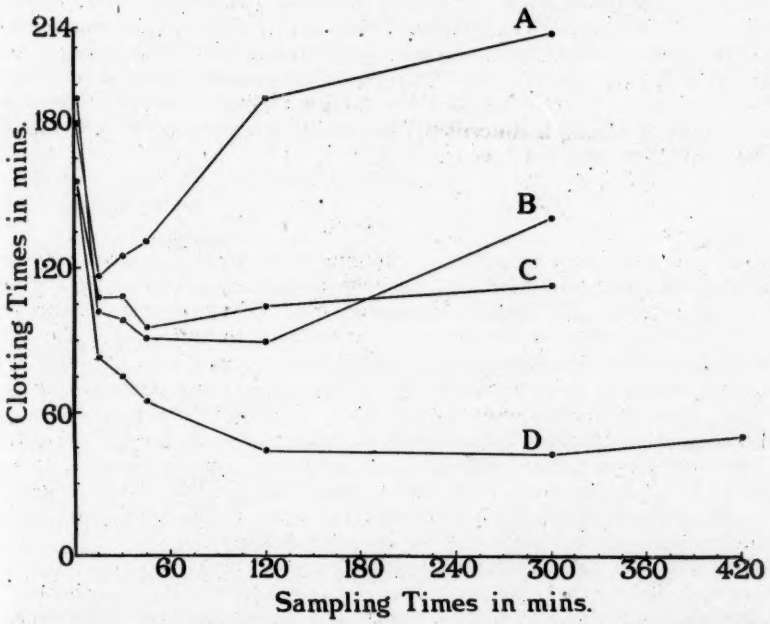

FIG. 1.—The effects of temperature and time on the activation of coagulase. Mixtures of coagulase and testis extract tested at intervals on citrated guinea-pig plasma. A = Mixture held at 37° C. tested at 37° C. B = Mixture held at 37° C. tested at 20° C. C = Mixture held at 20° C. tested at 37° C. D = Mixture held at 20° C. tested at 20 °C.

samples were withdrawn at intervals and added to standard quantities of citrated guinea-pig plasma diluted 1 in 10, one at 37° C., the other at room temperature. In the graph sampling times are plotted against clotting times. For reasons already discussed the reduction of clotting times, below that given by the sample withdrawn immediately after mixing coagulase and activator together, may be taken as an index of the amount of preformed thrombin-like product. This amount is determined by the relation between speeds of formation and destruction, which are chiefly dependent upon the temperature. Further formation and destruction proceed to a lesser degree after addition of samples to plasma so long as the samples still contain uncombined reagents but, by careful selection of the initial concentrations of coagulase and activator, this complication can be reduced to negligible proportions. It accounts, however, for the differences between coagulation times of the immediate samples. During the early stages of reaction, rate of formation exceeds rate of destruction so that clotting times are reduced and all the curves fall steeply and show relatively little divergence. Sooner or later, however, production and destruction balance each other, and still later the rate of destruction predominates, so that the curves flatten out and finally begin to rise. The effect of temperature on this production-destruction ratio is reflected in the divergence of the curves as time proceeds; at 37° C. destruction soon predominates, but at 20° C. this phase of the reaction is long delayed. Similar experiments have been done with different concentration of reagents, with mouse plasma, and with rabbit testis as the activator; although the ranges of clotting times have differed, as was to be expected, the results have been consistent.

The failure of coagulase to clot mouse and fowl plasmas at any temperature and the peculiar behaviour of guinea-pig plasma in clotting slowly at 20° C. whilst failing to do so at 37° C. are therefore explicable. With mouse and fowl plasmas the complete absence of activator leaves the coagulase quite inert. With guinea-pig plasma the formation of thrombin-like product from coagulase proceeds very slowly because of the low concentration of activator, but the thermolability of this product prevents it from ever attaining a concentration adequate for clotting if the test is made at 37° C., although at 20° C. clotting may occur.

DISCUSSION.

The conception of coagulase activity emerging from our experiments may be stated briefly as follows. Coagulase by itself is inert as far as conversion of fibrinogen to fibrin is concerned. It is, however, the precursor of a thrombin-like substance which can convert the fibrinogens of all species so far tested into fibrin clot. The formation of thrombin-like substance requires the participation of an activator; this is present in adequate quantity in some plasmas, but is deficient or completely lacking in others. Even within a single species individual variations occur. The reaction is therefore analogous to normal thrombin formation from prothrombin by the agency of thrombokinase, but with the important difference that calcium is not required. It would be more logical to apply the term coagulase to the thrombin-like clotting agent and to name the staphylococcus product pro-coagulase, but we have refrained from introducing such a change of nomenclature to avoid confusion.

This hypothesis is supported by all the relevant experimental evidence of which we are aware, including that supplied by previous workers who have suggested different interpretations. According to Gengou (1933) and Vanbreuseghem (1934), coagulase is either itself a fibrinolysin, or is associated with a fibrinolysin which is active at 37° C., but not at 20° C. They explain the failure of guinea-pig plasma to clot at the higher temperature, and also the non-coagulability of purified human fibrinogen, as being

due to the lysis of fibrin as quickly as it is formed. This necessitates the postulation of an anti-fibrinolysin in those plasmas which clot at 37° C. We have been unable to demonstrate the participation of any fibrinolysin in the coagulase reaction. Moreover a mixture of guinea-pig plasma and coagulase which has remained fluid during incubation at 37° C. for 24 hours will clot if placed at 20° C., showing that the fibrinogen has remained unchanged. A similar mixture which has been allowed to clot at 20° C. shows no subsequent lysis of the clot when placed at 37° C. even if additional coagulase, presumed to contain the fibrinolysin, is added. In our experience the subsequent disappearance of formed clot, which may occur when living staphylococci have been employed, is always associated with the production of acid; fibrin clot very rapidly disintegrates if the reaction is adjusted to below pH6.

The clotting of citrated plasma by coagulase is considerably slower than the normal clotting induced by addition of calcium chloride. Similarly coagulation of a solution of fibrinogen is more rapid on the addition of kinase and calcium chloride than by coagulase and activator. This is probably due to the very intimate association of normal prothrombin and fibrinogen, an association which is not ruptured even by repeated precipitations of the complex in attempts to obtain the fibrinogen in a pure state. The formation of normal thrombin must therefore take place in molecular intimacy with the fibrinogen, whereas the thrombin-like substance formed from coagulase must first attain such intimacy before it can fulfil its function.

The nature of the activator is not yet known. Testis extracts were used because at first it seemed probable that the agent might be thrombokinase; this, however, is not so. It has been found possible to remove practically all the kinase from plasma by dialysis without removing coagulase activator; on the other hand, activator is destroyed by heat treatment in the absence of electrolytes, leaving kinase unaffected. It may even be that the so-called activator is itself the substrate, and that substances other than coagulase exist capable of converting it into the thrombin-like product. Attempts are now under way to obtain concentrated activator freed from other tissue constituents like kinase which produce harmful effects on injection into animals. If these prove successful a new line of approach will be opened for the experimental investigation of the major problem of what part, if any, is played by coagulase in staphylococcal infections.

SUMMARY.

(1) Coagulase is a thermostable substance, either itself particulate or associated with particles of fairly uniform size. It is therefore readily filterable through Gradocol membranes of suitable porosity.

(2) Coagulase is the precursor of a thrombin-like substance, the production of which depends upon the participation of an activator present in some plasmas but deficient or totally lacking in others.

(3) The thrombin-like substance is thermolabile and its concentration at any moment depends upon several factors, chief of which are temperature, time, and the concentrations of reagents.

(4) The non-coagulability of the plasmas of some species and of occasional human beings in routine coagulase tests is due to deficiency of activator, or to conditions which prevent the accumulation of sufficient thrombin-like substance.

Acknowledgment: The expenses of this work were defrayed from a grant by the Medical Research Council.

REFERENCES.

CRUICKSHANK, R.—(1937) *J. Path. Bact.*, **45**, 295.
ELFORD, W. J.—(1931) *Ibid.*, **34**, 505.
GENGOU, O.—(1933) *Ann. Inst. Pasteur*, **51**, 14.
GROSS, H.—(1931) *Zbl. Bakt.*, **122**, 354.—(1933) *Klin. Wschr.*, **12**, 304.
LOEB, L.—(1903) *J. med. Res.*, **10**, 407.
MELLANBY, J.—(1908) *J. Physiol.*, **38**, 28.—(1909) *Ibid.*, **38**, 441.—(1930) *Proc. Roy. Soc.*, B, **107**, 271.—(1933) *Ibid.*, B, **113**, 93.
VANBREUSEGHEM, R.—(1934) *C. R. Soc. Biol.*, **116**, 344.—(1934) *Ibid.*, **116**, 650.
WALSTON, H. D.—(1935) *J. Hyg., Camb.*, **35**, 549.